Significance of steroidogenic enzymes in the pathogenesis of adrenal tumour

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We examined both activities and amounts of steroidogenic cytochrome P-450s at the posttranslational protein level and steroid contents in the adrenocortical adenoma from patients with primary aldosteronism and Cushing’s syndrome. Aldosterone synthase cytochrome P-450 (human P-450ald) was detected in the tumour portion of aldosterone-producing adenoma, but not in the normal control adrenals, at the protein level. Neither the activities nor the amounts of other P-450s in the tumour portion of aldosterone-producing adenoma were significantly different from those in the non-tumour portion in the adenoma and the normal control adrenals. The aldosterone content was significantly elevated, while the androstenedione content was significantly decreased in the tumour portion of the adenoma compared with that in the normal control adrenals. In Cushing’s syndrome, both the activities and amounts of P-45017α, and P-45011β were significantly elevated in the tumour portion compared with the non-tumour portion of the adenoma and the normal control adrenals, while those of P-45017β, and P-45011α in the tumour portion were not significantly different from the normal control adrenals. The cortisol content was significantly elevated, while the amounts of aldosterone and 18-hydroxysteroid oxidocorticosterone in the tumour portion of the adenoma were significantly decreased compared with those in the normal control adrenals. These results demonstrate that overexpression of P-450ald in aldosterone-producing adenoma, and those of P-45017α, and P-45011β in cortisol-producing adenoma may play some role in the pathogenesis of primary aldosteronism and Cushing’s syndrome, respectively.

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Primary aldosteronism of Cushing’s syndrome are hyperfunctioning adrenocortical adenomas which produce excessive amounts of aldosterone and cortisol, respectively. On the other hand, several enzyme defects, such as 21-hydroxylase and 11β-hydroxylase, in the adrenal gland have been demonstrated in congenital adrenal hyperplasia. On the basis of these findings, the hypothesis that a hyperfunctioning adrenal tumour is related to the enzymatic dysregulation should be examined. There are four major steroidogenic cytochrome P-450s involved in adrenocortical steroidogenesis, i.e. P-450c11β, and P-450c17β in the inner mitochondrial membranes, and P-450c17α and P-450c11β in the microsomal membranes of the adrenocortical cells. The aldosterone synthase cytochrome P-450 (human P-450ald), which was characterized in our laboratory (1) and others (2, 3), has been detected only in the mitochondria of the tumour portion of aldosterone-producing adenoma, not in those of the normal control adrenals at the protein level. Ogo et al. (4) found steroidogenic P-450 mRNAs in adrenocortical adenoma from patients with primary aldosteronism and suggested that the overproduction of aldosterone in aldosterone-producing adenoma resulted from increased expression of P-450c17β mRNA and decreased expression of P-450c11β mRNA. In the adrenal adenomas of Cushing’s syndrome, 21-hydroxylase activity was found to be significantly greater than in the normal adrenal tissue (5). Others have reported that 11β-hydroxylase, 17α-hydroxylase, and 21-hydroxylase activities were within the normal control range (6, 7). Ogo et al. (8) indicated that overproduction of cortisol in adrenocortical adenomas from patients with Cushing’s syndrome may result from increased expression of cytochrome P-450c11β and P-450c17α mRNA. Recent reports by Ogo et al. (4, 8) suggest that abnormal expression of the steroidogenic P-450 mRNAs may play a part in the pathogenesis of primary aldosteronism and Cushing’s syndrome. Nevertheless, neither the amounts of steroidogenic enzyme at the posttranslational protein level nor the enzyme activities have been determined, and it is necessary to determine the significance of the steroidogenic enzyme in the pathogenesis of the adrenal tumour. Furthermore, it is interesting to analyse the steroidogenic enzyme expression in the non-tumour portion of the hyperfunctioning adrenal tumour from the aspect of tumourogenesis. Although the previous studies have dealt with the adrenals of patients with renal cell carcinoma and breast cancer as the control, the present study compared the tumour portion of the hyperfunctioning gland with the normal adrenals of...
other patients with renal cell carcinoma as well as with the non-tumour portion of the hyperfunctioning gland with the same patient. This design would enable us to discuss the absolute differences of expressed steroidogenic enzyme expression between the hyperfunctioning adrenal gland and the normal ones. We determined the amounts and activities of the steroidogenic enzymes in the tumour and non-tumour portions of the adrenals separately.

**Patients and methods**

**Patients**

Adrenals of four patients with primary aldosteronism, four patients with Cushing’s syndrome and the normal control resected from three patients with renal cell carcinoma were used in the study. The clinical data are given in Table 1. The estimations of urinary 17-OHCS (17-hydroxycorticosteroids) and 17-KS (17-ketosteroids) were determined by the urinary metabolites of the serum adrenocortisol hormones. The urinary metabolites were measured by the urine hydrolysis with β-glucuronidase, followed by extraction and colorimetric quantitation by Porter-Silver (for 17-OHCS) and Zimmermann reaction (for 17-KS). Of the patients with primary aldosteronism, two were men (33 and 47 years old) and two were women (27 and 46 years old). Significantly higher levels of serum aldosterone and suppressed plasma renin activity (PRA) were obtained in all patients. Of the patients with Cushing’s syndrome, three were women (29, 33, and 34 years old) and one was a 45-year-old man. The levels of serum cortisol were significantly higher than those in normal subjects, and none of the four patients showed diurnal rhythm of serum cortisol level, or suppression of serum cortisol and urinary 17-OHCS by administration of either 2 or 8 mg of dexamethasone. A 55-year-old woman, who suffered from left deoxycorticosterone (DOC)-producing adrenal tumour, revealed hypertension and a hypermineralocorticoid state, i.e., high levels of peripheral (1.0 μg/l; normal value <0.13 μg/l) and the left adrenal venous DOC (19.5 μg/l versus 1.05 μg/l at the right side), suppressed PRA (0.06 ng·l⁻¹·s⁻¹), normal plasma aldosterone (98 ng/l) concentration, and low level of serum potassium (2.8 mmol/l). Furthermore, reversibility of the above-mentioned state by adrenalectomy point to the DOC-producing adenaoma. In the three control individuals with normal adrenals, no marked endocrinological abnormalities were detected.

**Measurement of enzyme activities**

Mitochondrial and microsomal fractions were obtained from the tumour and non-tumour portions of adrenals of all patients and the control adrenals by differential centrifugation (9).

**P-450 oxidase activity assay.** The mitochondrial fraction (30 μg of protein) was disrupted by freezing and thawing. In a reconstituted enzyme system including 111 μmol/l 20β-hydroxycholesterol as a substrate, 14 μmol/l adrenodoxin and 0.3 μmol/l NADPH-adrenodoxin reductase as an electron transfer system, 5 mmol/l isocitrate, 0.4 units isocitrate dehydrogenase per ml, and 5 mmol/l MgCl₂, as a NADPH-generating system were dissolved in 20 mmol/l potassium phosphate buffer (pH 7.4) and 0.3% TWEEN-20. The reaction was initiated by adding NADPH (final concentration 0.5 mmol/l) and terminated by adding 10% cholic acid. The product, pregnenolone, was converted to progesterone by adding cholesterol oxidase, and the final product was extracted with dichloromethane and analysed by HPLC on a TSK gel silica 150 column (4.6 × 250 mm; Tosoh, Tokyo, Japan)

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**Table 1. Clinical features of patients with primary aldosteronism, Cushing’s syndrome, and DOC-producing adenaoma.**

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Blood pressure (mmHg)</th>
<th>PRA (ng·l⁻¹·s⁻¹)</th>
<th>PAC (ng/l)</th>
<th>Serum Cortisol (μmol/l)</th>
<th>ACTH (ng/l)</th>
<th>Urinary 17-OHCS (mg/day)</th>
<th>Urinary 17-KS (mg/day)</th>
</tr>
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<td>1</td>
<td>27</td>
<td>Female</td>
<td>203/100</td>
<td>0.06</td>
<td>520</td>
<td>3.3</td>
<td>0.31</td>
<td>15</td>
<td>4.2</td>
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<td>2</td>
<td>47</td>
<td>Male</td>
<td>160/90</td>
<td>0.11</td>
<td>483</td>
<td>3.2</td>
<td>0.29</td>
<td>29</td>
<td>5.0</td>
</tr>
<tr>
<td>3</td>
<td>33</td>
<td>Male</td>
<td>180/100</td>
<td>0.06</td>
<td>596</td>
<td>2.7</td>
<td>0.45</td>
<td>20</td>
<td>6.5</td>
</tr>
<tr>
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<td>46</td>
<td>Female</td>
<td>180/100</td>
<td>0.06</td>
<td>400</td>
<td>2.9</td>
<td>0.20</td>
<td>32</td>
<td>8.8</td>
</tr>
<tr>
<td>1</td>
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<td>Male</td>
<td>170/90</td>
<td>0.06</td>
<td>25</td>
<td>3.6</td>
<td>0.53</td>
<td>10</td>
<td>12.9</td>
</tr>
<tr>
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<td>Female</td>
<td>160/90</td>
<td>0.08</td>
<td>30</td>
<td>3.7</td>
<td>0.67</td>
<td>10</td>
<td>16.0</td>
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<tr>
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<td>Female</td>
<td>150/90</td>
<td>0.06</td>
<td>26</td>
<td>2.7</td>
<td>0.68</td>
<td>12</td>
<td>24.2</td>
</tr>
<tr>
<td>4</td>
<td>34</td>
<td>Female</td>
<td>160/100</td>
<td>1.08</td>
<td>187</td>
<td>3.5</td>
<td>0.73</td>
<td>&lt;3</td>
<td>22.5</td>
</tr>
</tbody>
</table>

with n-hexan: isopropanol (100:2) as the mobile phase (10). The HPLC system consisted of a Tri rotor II pump and a Uvidec-100-III detector (Jasco, Tokyo, Japan). We used 5 mmol of deoxycorticosterone acetate (DOCA) as an internal standard.

**Assay of P-450scc and P-450a of activity.** The mitochondrial fraction (30 μg) was disrupted by freezing and thawing. In a reconstituted enzyme system including 100 μmol/L DOC as a substrate, 14 μmol/l adrenodoxin, 0.3 μmol/l NADPH-adrenodoxin reductase as an electron transport system, 5 mmol/l isocitrate, 0.4 units of isocitrate dehydrogenase per ml, and 5 mmol/l MgCl2 as a NADPH-generating system were dissolved in 100 mmol/L potassium phosphate buffer (pH 7.4) and 0.1 mmol/l EDTA. The reaction was initiated by adding NADPH (final concentration 0.5 mmol/l) and terminated by adding dichloromethane. The reaction product was extracted with dichloromethane and analysed by HPLC on a TSK gel silica 150 column with dichloromethane: ethanol: water (96:3:6:0.4) as the mobile phase (11, 12).

**Assay of P-450P1 and P-450scc activity.** The microsomal fraction (30 μg of protein) was reacted with 100 μmol/l progesterone as a substrate, 5 mmol/l isocitrate, 0.4 units of isocitrate dehydrogenase per ml, and 5 mmol/l MgCl2 as a NADPH-generating system, resolving into 100 mmol/L potassium phosphate buffer (pH 7.4) and 0.1 mmol/l EDTA. The reaction was initiated by adding NADPH (final concentration 0.5 mmol/l) and terminated by adding dichloromethane. The reaction product was extracted with dichloromethane and analysed by HPLC on a TSK gel silica 150 column with n-hexane: isopropanol: acetate (93:7:1) as the mobile phase (13). We used 10 nmol of spironolactone as an internal standard.

**Immunoblot analysis**

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of mitochondrial and microsomal lysates from human adrenal tumour was performed on 7.5% gels according to Laemmli’s method (14). Immunoblotting onto polyvinylidene difluoride membranes was performed as described previously (15). The membranes for each tumour were blocked with skim milk, incubated with anti-bovine P-450scc, anti-bovine P-45011β, anti-porcine P-45017β, or anti-bovine P-450c21 IgG (anti-bovine P-45011β IgG was a generous gift from Dr F. Mitani; the other products were purchased from OXYgene DALLAS), and then reacted with anti-rabbit IgG-biotin complex and visualized by reaction with diaminobenzidine (16).

**Measurement of steroid content of adrenal tumour tissue**

The steroid contents of the adrenals of the four patients with primary aldosteronism, three of the patients with Cushing’s syndrome, and the amounts of normal control adrenals were measured. The amounts of pregnenolone (Preg), DOC, corticosterone (B), 11-deoxy cortisol (11-DOF), dehydroepiandrosterone (DHEA), and androstenedione were measured by RIA by using, respectively, antisera against Preg-3-succinate-BSA, DOC-3-(O-carboxymethyl) oxime-BSA, B-3-(O-carboxymethyl) oxime-BSA, 11-DOF-3-(O-carboxymethyl) oxime-BSA. \( \Delta^2 \)-androstenedione-3-(O-carboxymethyl) oxime-BSA, preceded by extraction with ether (Teikoku Hormone Mg, Tokyo, Japan). The amounts of 18-hydroxydeoxycorticosterone (18-OH-DOC) were measured by RIA by using an antiserum against 18-OH-DOC-3-(O-carboxymethyl) oxime-BSA preceded by extraction with dichloromethane (Teikoku Hormone Mg). The cortisol content (F) was measured with an RIA kit (Baxter \( \gamma \) coat cortisol kit, USA). The progesterone (Prog) content was measured with another RIA kit (DPC progesterone kit, Japan DPC Co, Tokyo, Japan). The aldosterone content was measured with an Aldosterone RIA kit II (Dainabot Co, Tokyo, Japan).

**Results**

**Steroidogenic P-450 activities and amounts in primary aldosteronism. Cushing’s syndrome.** DOC-producing adenoma and the normal control adrenals

In humans, two enzymes, P-450scc and P-450a, are considered to be involved in aldosterone biosynthesis. The 48.5 kDa band, which moves slightly faster than P-45011β (50 kDa) in the immunoblot analysis with antibodies P-450scc IgG, is found to correspond to P-450a as well as in the rat (1). In primary aldosteronism, this 48.5 kDa protein was expressed only in the tumour portion of aldosterone-producing adenoma, as shown previously (1) (Figs. 1 and 2). The expressed amounts and activities of P-450scc, P-45011β, P-45017β, and P-450c21 in the tumour portion of aldosterone-producing adenoma shown to be similar to those in the normal tumour portion of the adenoma and the normal control adrenals (Figs. 1 and 2).

In Cushing’s syndrome, both P-450scc and P-450c21 activities were significantly elevated in the tumour portion of cortisol-producing adenoma by approximately threefold compared to those in the control adrenals. In concert with these changes, expressed amounts of these enzymes were elevated in the tumour portion (Fig. 2). Neither the activities nor amounts of P-450scc and P-45011β in cortisol-producing adenoma were significantly different from those in the control adrenals. Both activities and amounts of P-450scc, P-45017β, and P-450c21 in the non-tumour portion of the adenoma were significantly lower than in the tumour portion of the adenoma.

In DOC-producing adenoma, both activities and amounts of cytochromes P-450scc, P-45011β, and P-450c21 in the tumour portion of the adenoma were one-
third, one-third, and one-sixth of those in the non-tumour portion of the adenoma and the normal control adrenals, while those of other P-450s in the tumour portion were not significantly different from normal control adrenals.

**Steroid content of adrenals in primary aldosteronism, Cushing’s syndrome, and the normal control adrenals**

The steroid contents of two kinds of hyperfunctioning adrenal tumour are shown in Table 2. In aldosterone-producing adenoma, the aldosterone content was much higher (14-fold) than in the normal control adrenals, while the androstenedione content was significantly decreased, by approximately one-fourth.

In cortisol-producing adenoma, the cortisol content was significantly elevated, by 2.3-fold, while the amounts of 18-hydroxydeoxycorticosterone and aldosterone were significantly decreased compared with the normal control adrenals by one-fourth and one-twenty-fifth, respectively.

**Discussion**

The present study examined dysregulation of the steroi
dogenic P-450s at the posttranslational protein level in primary aldosteronism and Cushing’s syndrome. In the tumour portion of aldosterone-producing adenomas, aldosterone synthase cytochrome P-450 (P-450aldo), a recently identified enzyme, was found to be specifically overexpressed as we recently reported (1). Furthermore, both amounts and activities of cytochrome P-450c17, P-450c21, and P-450c21 in the tumour portion of the adenoma were similar to those in the non-tumour portion of aldosterone-producing adenoma and the normal control adrenals. In primary aldosteronism there is partial ACTH dependency in aldosterone biosynthesis, and plasma ACTH levels are not suppressed. The cytochrome P-450s responsible for the biosynthesis of steroid hormones are regulated in part by ACTH, which acts intracellularly through cAMP to increase gene transcription (17, 18). Therefore, it is reasonable that there are no differences in enzyme activities and amounts of P-450c17, P-450c21 between the non-tumour and the tumour portion of aldosterone-producing adenoma. Furthermore, the aldosterone content was significantly increased but the androstenedione content was decreased in the tumour portion, compared with those in the non-tumour portion of aldosterone-producing adenoma and normal control adrenals. Our findings on the steroid content of aldosterone-producing adenoma and cortisol-producing adenoma were essentially compatible with those reported by Kaplan et al. (19), Biglieri et al. (20), Ogo et al. (4, 8), and other investigators.

Ogo et al. (4) reported overproduction of aldosterone...
Fig. 2. Immunoblot analyses of enzyme amounts of P-450_{scc}, P-450_{11β}, P-450_{aldo}, P-450_{17α}, and P-450_{c21} from adrenals of patients with primary aldosteronism (PA), Cushing’s syndrome (CS), DOC-producing adenoma (DOC), and normal control adrenals (NL). T and N represent the tumour and non-tumour portions of the adrenal adenoma. In PA, the 48.5 kD band, which moves slightly faster than P-450_{11β} (50 kD band) in the immunoblot analysis with anti-bovine P-450_{11β} IgG, is found to correspond to P-450_{aldo} as well as in the rat (1). Numbers 1 to 3 point to individual cases.
Table 2. Steroid contents in the normal control adrenals, aldosterone-producing adenoma, and cortisol-producing adenoma.

<table>
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<tr>
<th>Cases</th>
<th>Preg (ng)</th>
<th>Prog (ng)</th>
<th>DOC (ng)</th>
<th>B (ng)</th>
<th>18-OH-DOC (ng)</th>
<th>11-DOF (ng)</th>
<th>F (μg)</th>
<th>Ald (ng)</th>
<th>DHEA (ng)</th>
<th>Andn (ng)</th>
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<td>3371 ± 1511</td>
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<td>1584 ± 422</td>
<td>43 ± 10³</td>
<td>2.2 ± 1.1³</td>
<td>622 ± 430</td>
<td>1329 ± 500</td>
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</tbody>
</table>

Average data are mean ± SEM. *p < 0.01 vs normal control adrenals, b*p < 0.05 vs normal control adrenals, respectively.

Preg (pregnenolone), Prog (pregosterone), DOC (doxycorticosterone), B (corticosterone), 18-OH-DOC (18-hydroxydoxycorticosterone), 11-DOF (11-deoxycortisol), F (cortisol), Ald (aldosterone), DHEA (dehydroepiandrosterone), Andn (androstenedione).
in adrenocortical adenomas from patients with primary aldosteronism resulting from increased expression of P-450<sub>17β</sub> mRNA and decreased expression of P-450<sub>17α</sub> mRNA. Their finding of increased expression of P-450<sub>11β</sub> mRNA may point to mRNA of P-450<sub>aldo</sub> which we have purified.

Recently we also reported that both angiotensin II and high levels of extracellular potassium induced significant increases in both amount and activity of aldosterone synthase cytochrome P-450 (P-450<sub>aldo</sub>) in the zona glomerulosa mitochondria of rat adrenals (21). The syndrome of primary aldosteronism is characterized by excessive production of aldosterone despite normal plasma ACTH level, suppression of PRA, and low levels of serum potassium. Taken together, these findings indicate that aldosterone-producing adenoma cells produce aldosterone autonomously, and our results suggest that overexpression of P-450<sub>aldo</sub> not P-450<sub>11β</sub> may be one of the candidates for the pathogenesis of primary aldosteronism, but it is likewise possible that dysregulation of P-450<sub>aldo</sub> expression may constitute a manifestation of tumour development of aldosterone-producing cells. According to the histological findings of Dhom and Städtler (22), the surrounding tissues of different patients with primary aldosteronism show differences as they were histologically studied, for example, atrophy, hyperplasia, hyperplasia with micronodulation. As we measured both activities and amounts of steroidogenic enzymes in the mixed lysate of mitochondrial and microsomal fraction from the adrenals of the patients, and we did not perform histochemical and immunohistochemical studies of steroidogenic enzymes, we could not discuss parallelly between histological and steroid findings.

In Cushing's syndrome, both enzyme activities and amounts of P-450<sub>17α</sub> and P-450<sub>c21</sub> were found to be significantly greater in the tumour portion of cortisol-producing adenoma, than in the non-tumour portion of the adrenal and in normal control adrenals, while those of P-450<sub>cc</sub> and P-450<sub>11β</sub> were unchanged.

Evidence that both enzyme activities and amounts of P-450<sub>cc</sub>, P-450<sub>17α</sub>, and P-450<sub>c21</sub> were significantly more suppressed in the non-tumour portion than in the tumour portion may be the result of suppression of the plasma ACTH level by negative feedback inhibition of cortisol overproduction. These results suggest that overexpression of both P-450<sub>17α</sub> and P-450<sub>c21</sub> may be responsible for the pathogenesis of Cushing's syndrome. Ogo et al. (8) reported that overproduction of cortisol in adrenocortical adenomas from patients with Cushing's syndrome may result from markedly increased expression of cytochrome P-450<sub>17α</sub> and P-450<sub>c21</sub> mRNA. Taken together, our results confirm that overexpression of both cytochrome P-450<sub>17α</sub> and P-450<sub>c21</sub> play some role in autonomous production of cortisol in Cushing's syndrome at the transcriptional level as well as at the posttranslational level.

In DOC-producing adenoma, both activities and amounts of cytochromes P-450<sub>17α</sub> and P-450<sub>11β</sub> in the tumour portion of the adenoma were one-third of those in the non-tumour portion of the adenoma and the normal control adrenals. These data demonstrated that underexpression as well as overexpression of steroidogenic enzymes play some role in the pathogenesis of hyperfunctioning adrenal tumour.

In conclusion, hyperfunctioning adrenal tumour may be the result of abnormal expression of steroidogenic enzymes; however, factors which affect expression of the enzymes should be clarified.

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